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TITLE OF THE INVENTION (280 characters max)

USE OF GENETIC POLYMORPHISMS TO PREDICT DRUG-INDUCED HEPATOTOXICITY

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USE OF GENETIC POLYMORPHISMS TO PREDICT DRUG-INDUCED HEPATOTOXICITY

FIELD OF THE INVENTION

[0001] This invention relates generally to the analytical testing of tissue samples *in vitro*, and more particularly to the analysis of genetic polymorphisms as biomarkers for predicting the occurrence of drug-induced hepatotoxicity.

BACKGROUND OF THE INVENTION

[0002] Among the disorders that arise from dysfunction of the microvasculature of diabetic patients is retinopathy, which manifests itself clinically as vision impairment and can result in blindness. Diabetic retinopathy is characterized by microaneurysms, excessive vascular permeability, areas of retinal nonperfusion, and retinal neovascularization. Much evidence suggests a causal link between high blood glucose levels and the development of the underlying lesions responsible for deficits in organ function. For a review, see Way KJ *et al., Diabetic Medicine* 18: 945-59 (2001).

[0003] Among the effects of hyperglycemia is the over-activation of the diacylglycerol (DAG) – protein kinase C (PKC) signal transduction pathway. Both cell culture experiments and animal models of diabetes demonstrate excessive levels and activity of DAG and PKC in vascular endothelial cells. Koya D & King GL, *Diabetes* 47: 859-866 (1998); Ishii H *et al., J Mol Med* 76: 21-31 (1998) and Way KJ *et al., Trends Pharmacol Sci* 21: 181-7 (2000). Activation of many of the isoforms of PKC serine-threonine kinases is dependent on DAG, a cleavage product of membrane phospholipids. Among the activated isoforms of PKC serine-threonine kinases is the predominant isoform PKCβ, which is associated with diabetic retinopathy. DAG is usually generated by agonist-stimulated hydrolysis of membrane phospholipids, but also can be synthesized *de novo* by direct metabolism of glucose. Dunlop ME & Larkins RG, *Biochem Biophys Res Commun* 132: 467-73 (1985); Ishii H *et al., J Mol Med* 76: 21-31 (1998). In response to hyperglycemia, *de novo* synthesis of DAG increases substantially, resulting in the activation of PKCβ. Ishii H *et al., J Mol Med* 76: 21-31 (1998).

[0004] As a consequence of continual activation of the DAG-PKC pathway, many aspects of vascular function are affected. Cytokine activation and leukocyte adhesion are stimulated; blood flow and microvessel contractility are altered; and extracellular matrix synthesis increases, resulting in the thickening of basement membranes. The retinal microenvironment becomes ischemic as a result of the aforementioned changes. Expression of vascular endothelial growth factor (VEGF), a potent stimulator of neovascularization, is upregulated in response to ischemia and by other PKCβ-dependent mechanisms. Aiello LP *et al., Diabetes* 46: 1473-80 (1997).

[0005] N-benzoyl-staurosporine (PKC412) is an inhibitor of both PKC and an essential VEGF receptor, KDR (Kinase insert Domain-containing Receptor, also known as VEGF-R2). N-benzoyl-staurosporine is being developed for several indications, including the treatment of diabetic macular edema. See, U.S. Pat. No. 6,214,819. See also, U.S. Pat. Appln. 20030119812, 20030125343 and 20030153551. Although a promising medication, treatment with N-benzoyl-staurosporine can result in known side effects, including liver toxicity. Thus, there is a need in the art for reducing the side effects of side effects of N-benzoyl-staurosporine.

SUMMARY OF THE INVENTION

[0006] The invention provides methods for determining subjects who are at risk for developing drug-induced hepatotoxicity. In one embodiment, the invention provides for the use of genomic analysis to identify patients at risk for experiencing hepatotoxicity during staurosporine therapy. In a particular embodiment, the staurosporine therapy involves the administration of N-benzoyl-staurosporine for treating diabetic macular edema. The hepatotoxicity prediction involves the determination of serum aspartate transaminase (AST) levels. In another embodiment, the invention provides methods for determining optimal treatment strategies for these patients.

[0007] The invention also provides clinical assays, kits and reagents for predicting hepatotoxicity prior to taking a drug. In one embodiment, the kits contain reagents for determining genetic polymorphisms in the *IL1A* gene. In a particular embodiment, the genetic polymorphism is at the PG locus ID 279 of the *IL1A* gene. In assays of genetic polymorphism of PG locus ID 279, the CC genotype is a biomarker for predictions of higher risk of hepatotoxicity, while the CT and TT genotypes are biomarkers for a lower risk of hepatotoxicity. In another

embodiment, the kits contain reagents for determining genetic polymorphisms in the *IL1A* gene. In a particular embodiment, the genetic polymorphism is at the PG locus ID 302 of the *IL1A* gene. In assays of genetic polymorphism of PG locus ID 302, the GG genotype is a biomarker for predictions of higher risk of hepatotoxicity, while the CT and TT genotypes are biomarkers for a lower risk of hepatotoxicity.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] FIG. 1 shows the AST/ALT (aspartate aminotransferase / alanine aminotransferase) maximum levels vs. *IL1A* (interleukin 1-alpha; PG locus ID 279). The scatter plots show (A) maximum aspartate aminotransferase levels, (B) ratio of ASTMAX and the upper limit of normal (ULN), (C) maximum alanine aminotransferase levels and (D) ratio of ALT MAX and ULN for subjects in the clinical trial with genotypes of CC or T (CT or TT) for IL1A PG locus ID 279. The upper limit of normal for aspartate aminotransferase is 42 U/L for ages 3-64 and 55 U/L for 65 and over and for alanine aminotransferase is 48 U/L for all ages. ULNs are indicated by a line.

[0009] FIG. 2 shows the AST/ALT (aspartate aminotransferase / alanine aminotransferase) maximum levels vs. IL1A (interleukin 1-alpha; PG locus ID 302). The scatter plots show plots of (A) maximum alanine aminotransferase levels, (B) ratio of ASTMAX and ULN, (C) maximum ALT levels and (D) ratio of ALT MAX and ULN for subjects in the clinical trial with genotypes of GG or T (CT or TT) for IL1A PG locus ID 302. The upper limit of normal for aspartate aminotransferase is 42 U/L for ages 3-64 and 55 U/L for 65 and over and for alanine aminotransferase is 48 U/L for all ages. ULNs are indicated by a line.

DETAILED DESCRIPTION OF THE INVENTION

[0010] The invention advantageously provides a way to determine whether a patient will experience hepatotoxicity during drug treatment, prior to actually taking the drugs. The invention thus provides safer treatment regimens for patients by helping clinicians to either (1) alter the dose of the drug, (2) provide additional or alternative concomitant medication or (3) choose not to prescribe that drug for that patient.

[0011] Relevant genetic polymorphisms were identified in a multicenter, randomized, double-masked placebo-controlled dose finding phase II trial of N-benzoyl-staurosporine, which was conducted in subjects with diabetic macular edema. The safety of N-benzoyl-staurosporine

was evaluated in the subjects and additional pharmacokinetic information on was collected. While N-benzoyl-staurosporine showed a good safety profile, nine of the 140 subjects that were enrolled in the clinical trial experienced hepatotoxicity, as defined by fold increases in liver transaminases over the upper limit of normal (ULN). Subjects were flagged as having experienced hepatotoxicity if either aspartate aminotransferase (AST) or alanine aminotransferase (ALT) had fold elevations over the upper limit of normal on visit 3, 4 or 5.

[0012] Of the eighteen single nucleotide polymorphisms (SNP) from seven genes that were genotyped, two in the *interleukin 1-alpha* (*IL1A*) gene were associated with the maximum serum aspartate transaminase level recorded on visits 3, 4 or 5. *IL1A* encodes an inflammatory cytokine that plays a pivotal role in mediating acute phase responses. One *IL1A* polymorphism is located in the promoter region of *IL1A* and the other results in a serine to alanine substitution at amino acid position 114. These results suggest that polymorphisms in *IL1A* or a gene located near it on 2q14, may be directly involved with the onset of liver toxicity following administration of N-benzoyl-staurosporine.

[0013] As used herein, a polymorphism in the *IL1A* genetic locus is "predictive" of a "high" risk of hepatotoxicity when genetic polymorphism correlates significantly with the development of drug-induced hepatotoxicity or with elevated levels of serum aspartate transaminase. See, for example, below, where the CC genotype at the PG locus ID 279 and the GG genotype at the PG locus ID 302 are predictive of a high risk of hepatotoxicity. As used herein, a polymorphism in the *IL1A* genetic locus is "predictive" of a "low" risk of hepatotoxicity when genetic polymorphism correlate significantly with the lack of development of hepatotoxicity. See, for example, below, where the CT or TT genotype at the PG locus ID 279 and the CT or TT genotype at the PG locus ID 302 are predictive of a low risk of hepatotoxicity. Determinations of significance (p values) can be determined by analysis of variance (ANOVA) or Fisher's Exact tests. Determinations of one SNP polymorphism at a certain IL1A genetic site as having a high risk for developing hepatotoxicity and another SNP polymorphism at that IL1A genetic site as having a low risk for developing hepatotoxicity can be combined for greater accuracy of determination. For PG locus IDs 279 and 302, associations between *IL1A* polymorphisms and serum aspartate transaminase levels had p values of 0.0089 and 0.0097.

[0014] These results can reasonably be extrapolated to the prediction of hepatotoxicity in patients following the administration of any staurosporine derivatives, based upon the structural

similarity and modes of action in the liver of staurosporine derivatives. Among the staurosporine derivatives are those described in U.S. Pat. No. 5,093,330. Preferred compounds are N-acylstaurosporines and their pharmaceutically acceptable salts, including N-(2-aminoacetyl)staurosporine; N-(3,5-dinitrobenzoyl)-staurosporine; N-(3-carboxypropionyl)staurosporine; N-(3-fluorobenzoyl)-staurosporine; N-(3-nitrobenzoyl)staurosporine; N-(4-carboxybenzoyl)staurosporine; N-[(tert-butoxycarbonylamino)-acetyl]-staurosporine; N-alanylstaurosporine; N-benzoyl staurosporine; N-carboxymethyl-staurosporine; N-ethyl-staurosporine; N-methylaminothiocarbonylstaurosporine; N-phenylcarbamoylstaurosporine; N-tert-butoxycarbonylstaurosporine; and N-trifluoracetylstaurosporine.

[0015] Moreover, the results can be extrapolated to the prediction of hepatotoxicity in patients who are being treated for diseases other than diabetic macular edema. The method of the invention is applicable to vertebrate subjects, particularly to mammalian subjects, more particularly to human subjects. The invention is particularly applicable to diabetic subjects.

[0016] The diagnosis of hepatotoxicity can be accomplished using assays of serum enzyme levels. Serum enzyme assays indicative of liver dysfunction are well-known to those of skill in the medical arts and routine in hospital laboratories. For a definition of hepatotoxicity based upon serum levels of aspartate transaminase (AST) and used in the EXAMPLE: The definition of hepatotoxicity used in this analysis was based on fold increases in serum aspartate transaminase or alanine aminotransferase over the upper limit of normal (ULN) on visits 3, 4 or 5. The upper limit of normal for serum aspartate transaminase is 42 U/L for ages 3-64 and 55 U/L for ages 65 and up; for serum alanine aminotransferase the upper limit of normal is 48 U/L (Smithkline Beecham Clinical Laboratories Reference Alert Ranges). While an elevation of either enzyme at visit 3, 4 or 5 constituted hepatotoxicity, transaminase elevations were disregarded during subsequent visits when the drug was not being administered. Furthermore, subjects who had elevated liver function tests at baseline (visit 2) were not flagged as having experienced hepatotoxicity, regardless of the elevation in their enzyme levels following administration of the drug. Nine subjects were flagged as having experienced hepatotoxicity. Of these, six consented to clinical pharmacogenetic analysis.

[0017] Individuals carrying polymorphic alleles may be detected at the DNA, the RNA, or the protein level using a variety of techniques that are well known in the art. Strategies for

identification and detection are described in *e.g.* EP 730,663, EP 717,113, and PCT US97/02102. The methods of the invention may involve the detection of pre-characterized polymorphisms. That is, the genotyping location and nature of polymorphic forms present at a site have already been determined (see, discussion above regarding interrogated genes). The availability of this information allows sets of probes to be designed for specific identification of the known polymorphic forms. The identification of alleles containing single nucleotide polymorphisms may involve the amplification of DNA from target samples. This can be accomplished by *e.g.*, PCR. See generally *PCR Technology: Principles and Applications for DNA* Amplification, (ed. Erlich, Freeman Press, New York, New York, 1992); *PCR Protocols: A Guide to Methods and Applications* (eds. Innis, *et al.*, Academic Press, San Diego, Calif., 1990). The detection of polymorphisms in specific DNA sequences, can be accomplished by a variety of methods including, but not limited to, restriction-fragment-length-polymorphism detection based on allele-specific restriction-endonuclease cleavage (Kan & Dozy, *Lancet* II:910-912 (1978)), hybridization with allele-specific oligonucleotide probes (Wallace *et al, Nucl. Acids Res.* 6:3543-3557 (1978)), including immobilized oligonucleotides (Saiki *et al., Proc. Natl. Acad. Sci. USA*, 86:6230-6234 (1969)) or oligonucleotide arrays (Maskos & Southern, *Nucl. Acids Res.* 21:2269-2270 (1993)), allele-specific PCR (Newton et *al., Nucl. Acids Res.* 17:2503-2516 (1989)), mismatch-repair detection (MRD) (Faham & Cox, *Genome Res.* 5:474-482 (1995)), binding of MutS protein (Wagner *et al., Nucl. Acids Res.* 23:3944-3948 (1995), denaturing-gradient gel electrophoresis (DGGE) (Fisher & Lerman, *Proc. Natl. Acad. Sci. U.S.A.* 80:1579-1583 (1983)), single-strand-conformation-- polymorphism detection (Orita *et al., Genomics* 5:874-879 (1983)), RNAse cleavage at mismatched base-pairs (Myers *et al., Science* 230:1242 (1985)), chemical (Cotton *et al., Proc. Natl. Acad. Sci. U.S.A.*, 8Z:4397-4401 (1988)) or enzymatic (Youil *et al., Proc. Natl. Acad. Sci. U.S.A.* 92:87-91 (1995)) cleavage of heteroduplex DNA, methods based on allele specific primer extension (Syvanen et *al., Genomics* 8:684-692 (1990)), genetic bit analysis (GBA) (Nikiforov *et al., Nucl. Acids Res.* 22:4167-4175 (1994)), the oligonucleotide-ligation assay (OLA) (Landegren *et al., Science 241*:1077 (1988)), the allele-specific ligation chain reaction (LCR) (Barrany, *Proc. Natl. Acad. Sci. U.S.A.* 88:189-193 (1991)), gap-LCR (Abravaya *et al., Nucl. Acids Res.* 23:675-682 (1995)), radioactive and/or fluorescent DNA sequencing using standard procedures well known in the art, and peptide nucleic acid (PNA) assays (Orum *et al., Nucl. Acids Res.* 21:5332-5356 (1993); Thiede *et al.*,

Nucl. Acids Res. 24:983-984 (1996)). Additional guidance is provided by Sambrook J *et al.*, *Molecular Cloning: A Laboratory Manual, Third Edition* (Cold Spring Harbor Press, Cold Spring Harbor, New York, 2000).

[0018] Guidance for the use of N-benzoyl-staurosporine and related staurospaurine derivatives is provided in U.S. Pat. Nos. 5,744,460; 5,827,846; 6,018,042; 6,153,599 and 6,214,819, each of which is incorporated by reference. Additional guidance for the use of N-benzoyl-staurosporine related staurospaurine derivatives for treating ocular neovascular diseases and in decreasing capillary permeability in the retina is provided in U.S. Pat. Applns. 20030119812, 20030125343 and 20030153551, each of which is incorporated by reference.

[0019] The kits of the invention may contain a written product on or in the kit container. The written product describes how to use the reagents contained in the kit to determine whether a patient will experience hepatotoxicity during drug treatment. In several embodiments, the use of the reagents can be according to the methods of the invention. In one embodiment, the reagents are primer pairs for performing PCR analysis of *IL1A* genetic polymorphisms.

EXAMPLE
CLINICAL PHARMACOGENETIC ANALYSIS OF HEPATOTOXICITY IN THE CLINICAL TRIAL

[0020] *Demographics of clinical pharmacogenetic analysis participants.* Of the 139 subjects enrolled in the clinical trial, 83 consented to participation in the clinical pharmacogenetic portion of the clinical trial. This represents about 60% of the total population that participated in the clinical trial. The clinical pharmacogenetic analysis population was representative of the clinical trial group in terms of age, race and gender. Furthermore, the consent rate was comparable for each arm of the trial (placebo, 50, 100 and 150 mg/day), such that the clinical pharmacogenetic analysis was not biased toward one dosage group. No statistically significant differences were observed between the demographics of the clinical pharmacogenetic population compared to the overall trial population.

Table 1
Distribution of clinical pharmacogenetic samples compared to the overall clinical trial samples

	CPG samples	Trial samples
AGE (mean,years)[a]	58.8	59.3
RACE[b]		
Caucasian	(70) 84%	(117) 84%
Black	(5) 6%	(9) 6.4%
Oriental	(1) 1.2%	(2) 1.4%
Hispanic	(7) 8.4%	(9) 7%
Other	(0) 0%	(2) 1.4%
GENDER[c]		
Male	(50) 60%	(88) 63%
Female	(33) 40%	(51) 37%
DOSE[d]		
0 mg/day	(23) 28%	(34) 24%
50 mg/day	(20) 24%	(32) 23%
100 mg/day	(20) 24%	(37) 27%
150 mg/day	(20) 24%	(36) 26%
Hepatotoxicity[e]		
Yes	(6) 7%	(9) 6%
No	(77) 93%	(130) 94%

[a] $p<0.7748$ (ANOVA)
[b] $p<0.9112$ (Fisher's Exact)
[c] $p<0.6698$ (Fisher's Exact)
[d] $p<0.9342$ (Fisher's Exact)
[e] $p<1.000$ (Fisher's Exact)

[0021] Blood samples from each patient were collected at individual trial sites and shipped to Covance (Geneva, Switzerland), where genomic DNA was extracted using the PUREGENE™ DNA Isolation Kit (D-50K) (Gentra, Minneapolis, MN).

[0022] *Genotyping*. A total of 18 loci in seven genes were genotyped. SNP assays were designed using information from public databases such as OMIM, the SNP Consortium, Locus Link and dbSNP, and information from Third Wave Technologies, Inc. (TWT, Madison, WI). Any loci that were not polymorphic in this trial population were not analyzed further. Genotyping was performed with 40-60 ng of genomic DNA using the Invader® assay developed by Third Wave Technologies according to the manufacturer's instructions. Lyamichev V *et al.,Nat Biotechnol* 17: 292-6 (1999); Ryan D *et al.. Mol Diagn* 4: 135-44 9 (1999)

[0023] Each SNP interrogated in this trial was assigned a clinical pharmacogenetic (CPG) identifier, referred to as the PG locus ID. See Table 2 for a list of all SNPs assayed in this trial, along with their PG locus IDs and details about the location of the polymorphism within the gene of interest.

Table 2
List of genes examined in the clinical pharmacogenetic analysis

Gene Symbol	Gene Name	CPG Locus ID	REF_ACC	Allele 1 Freq.	Allele 2 Freq.	Position	Location
ABCB1	*ATP-binding cassette, sub-family B (MDR/TAP), member 1*	181	M29445	0.47	0.53	176	Exon 26 ILE1144ILE
ABCB1	*ATP-binding cassette, sub-family B (MDR/TAP), member 1*	1006	AC005068.1	0.63	0.37	83646	Unknown
ABCB1	*ATP-binding cassette, sub-family B (MDR/TAP), member 1*	1045	AC002457.1	0.48	0.52	19981202	Unknown
CD14	*CD14 antigen*	1708	U00699	0.56	0.44	312	Promoter
CYP2D6	*cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6*	31	M33388	0.86*	0.14*	3465	Intron
CYP2D6	*cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6*	211	M33388	0.60*	0.40*	4469	Exon 6 ARG269CYS
CYP2D6	*cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6*	212	M33388	0.52	0.48	5799	Exon 9 THR486SER
CYP2D6	*cytochrome P450, subfamily IID (debrisoquine, sparteine, etc., -metabolizing), polypeptide 6*	213	M33388	0.83	0.17	1719	Exon 1 PRO34SER

Table 2
List of genes examined in the clinical pharmacogenetic analysis

Gene Symbol	Gene Name	CPG Locus ID	REF_ACC	Allele 1 Freq.	Allele 2 Freq.	Position	Location
CYP3A4	*cytochrome P450, subfamily IIIA (niphedipine oxidase), polypeptide 4*	2325	AF209389	0.84*	0.16*	20338	Intron 10
IL1A	*interleukin 1, alpha*	279	X03833	0.72*	0.28*	549	Promoter
IL1A	*interleukin 1, alpha*	302	X03833	0.72*	0.28*	6282	Exon 5
IL1A+	*interleukin 1, alpha*	303	X03833	1.00	0.00	4282	Exon 4
NR1I2	*nuclear receptor subfamily 1, group I, member 2*	2641	AC069444	0.62*	0.38*	42813	Promoter
NR1I2	*nuclear receptor subfamily 1, group I, member 2*	2642	AF061056	0.60	0.40	252	Intron
NR1I2	*nuclear receptor subfamily 1, group I, member 2*	2643	AF061056	0.80	0.20	11156	3' UTR
ORM1	*orosomucoid 1 (alpha-1-acid glycoprotein)*	1831	NT_031830	0.57*	0.43*	191	Exon 1
ORM1	*orosomucoid 1 (alpha-1-acid glycoprotein)*	1832	NT_031830	0.97	0.03	2077	Exon 5
ORM1	*orosomucoid 1 (alpha-1-acid glycoprotein)*	1833	NT_031830	0.96	0.04	5041	Intron

*This SNP is not in Hardy-Weinberg equilibrium in this patient population.
†This locus is not polymorphic in these patient populations and was not used in the analysis.

[0024] Loci in *ABCB1*, *CD14*, *IL1A*, and *ORM1* were interrogated by directly assaying genomic DNA using Third Wave Technologies technology. Because of high sequence homology within the CYP450 family of genes, *CYP2D6* was polymerase chain reaction (PCR) amplified in three fragments prior to Invader® assay genotyping to insure specificity. Primer sequences for each segment are listed in Table 3 along with the region of the gene spanned by each primer set. Each amplicon was generated in a 25 µl reaction containing 20-60 ng of genomic DNA, 0.5 µl of 10 mM dNTPs, 2.5 µl of 10X PCR Buffer I with 15 mM $MgCl_2$ (Applied Biosystems, Foster City, CA), 2.5 µl DMSO, 0.5 µl of 20 µM CYP2D6-forward primer, 0.5 µl of 20 µM CYP2D6-reverse primer, and 1.25 U Taq DNA polymerase (Applied Biosystems). 35 cycles of amplification were performed using the following conditions: 94°C, 30 sec; 65°C, 1 min.; 72°C, 2 min. Amplification of the appropriate product was confirmed on five random samples by

fractionation on a 1% agarose gel containing ethidium bromide. Amplicons were diluted 1:10 in TE (pH 8.0) before genotyping.

Table 3
Primers used to amplify CYP2D6

Gene region (PG locus ID)	Primer Name	Primer Sequence (5'→3')	Size (bp)
Exons 1 and 2 (31)	2D6L1F1	CTGGGCTGGGAGCAGCCTC (SEQ ID NO:1)	2036
	2D6L1R1	CACTCGCTGGCCTGTTTCATGTC (SEQ ID NO:2)	
Exons 3, 4 and 5 (31 & 211)	2D6L2F	CTGGAATCCGGTGTCGAAGTGG (SEQ ID NO:3)	1683
	2D6L2R2	CTCGGCCCCTGCACTGTTTC (SEQ ID NO:4)	
Exons 7, 8 and 9 (212)	2D6L3F	GAGGCAAGAAGGAGTGTCAGGG (SEQ ID NO:5)	1754
	2D6L3R5B	AGTCCTGTGGTGAGGTGACGAGG (SEQ ID NO:6)	

[0025] PCR for the *CYP3A4* gene was performed in a 25 µl reaction containing 15-30 ng genomic DNA, 0.4 µl of 10 mM dNTPs, 2.5 µl of 10X PCR BufferI with 15 mM $MgCl_2$ (Applied Biosystems), 0.75 µl of 20 µM CYP3A4-forward primer, 0.75 µl of 20 µM CYP3A4-reverse primer, and 0.75 U Taq DNA polymerase (Applied Biosystems). 30 cycles of amplification were performed using the following conditions: 94°C, 30 sec; 58°C, 30 sec; 72°C, 30 sec. To confirm that the appropriate product was generated, five samples were fractionated on a 1% agarose gel containing ethidium bromide and fragment size was visualized. Primer sequences are as follows: CYP3A4Exon10F-(5'-TGGATGGCCCACATTCTCG-3'; SEQ ID NO:7), and CYP3A4Exon10R-(5' CTTCCTACATAGAGTCAGTG-3'; SEQ ID NO:8). A 1:20 dilution of the PCR product in TE (pH 8.0) was run against PG locus ID 2325 using a 384 well biplex plate for amplified DNA.

[0026] Restriction Fragment Length Polymorphism (RFLP) analysis was used to genotype three polymorphic loci in the *NR1I2*. *NR1I2* sequences were first PCR amplified in 25 µl reactions containing 15-30 ng genomic DNA, 0.4 µl of 10 mM dNTPs, 2.5 µl of 10X PCR Buffer I with 15 mM $MgCl_2$ (Applied Biosystems), 0.50 µl of 20 µM NR1I2-forward primer, 0.50 µl of 20 µM NR1I2-reverse primer, and 0.75 U Taq DNA polymerase (Applied Biosystems).

Sequences for the primer sets used for each assay are listed in Table 4. Thirty-five cycles of amplification were performed using the following conditions: 94°C, 30 sec; 60°C, 30 sec; 72°C, 30 sec. Amplicons were fractionated on a 3% agarose gel containing ethidium bromide.

Table 4
RFLP analysis of *NR1I2* polymorphisms

PG locus ID (*Assay Name*)	Primer Name	Primer Sequence (5'-3')	Rstriction Endonuclease	Frag Size (bp)/ allele call
2642 (*PXR-252*)	PXR-252-F	GGACACAGAGTCTG TTCCTGG (SEQ ID NO:9)	BSMB1	318/A 204 & 114/G
	PXR-252-R	GAAGATGAAGGATT CCTCTGGG (SEQ ID NO:10)		
2643 (*PXR-11156*)	PXR-11156-F	GACAAGGCTACGCT GACAATCAG (SEQ ID NO:11)	DdeI	342/C 193 & 149/A
	PXR-11156-R	GCTTGCGTATGTTTC TATTTCCAC (SEQ ID NO:12)		
2641 (*PXR-24113*)	PXR-24113-F	CGGAGCAAAGAACT TACCACC (SEQ ID NO:13)	HphI	253/G 195 & 58/A
	PXR-24113-R	TGCAGGACCAGAGA GCATCAG (SEQ ID NO:14)		

[0027] Restriction enzymes used for RFLP analysis of the PCR products are listed in Table 4 along with the fragment size they produced and the resultant allele call. All restriction enzymes were purchased from New England Biolabs, Beverly, MA. Reaction conditions were as follows: (1) BMSB1 digests were performed in a 20 µl reaction using 2 µl of 10X Buffer 3 (New England Biolabs), 8 µl amplified DNA, and 2U BSMB1 enzyme. Reaction mixtures were incubated for 4.5 hours at 55°C; (2) DdeI digests were performed in a 20 µl reaction using 2 µl of 10X Buffer 3 (New England Biolabs), 8 µl amplified DNA, and 4 U of DdeI enzyme. Reactions were incubated for 17 hours at 37°C; (3) HphI digests were as DdeI digests except 10X Buffer 4 (New England Biolabs) was used instead of 10X Buffer 3. Digested DNA was fractionated (10 µl) on a 3% agarose gel containing ethidium bromide and band size was visualized.

[0028] *Statistical Analysis.* Analysis of variance (ANOVA) and Fisher's Exact tests were used for the analysis of the effect of genotype and hepatotoxicity. All statistical analyses were performed using the SAS 8.02 software. To correct for multiple testing, the Bonferroni correction method was performed (see, below).

[0029] *Hepatotoxicity and N-benzoyl-staurosporine metabolism.* The relationship between the occurrence of hepatotoxicity in the clinical trial and the dose of N-benzoyl-staurosporine administered was examined. The percentage of subjects per dose group that experienced hepatotoxicity are as follows: 3% for 50 mg/day, 8% for 100 mg/day and 14% of those that took 150 mg/day. While most of the subjects that experienced liver toxicity took the highest dose of N-benzoyl-staurosporine, the association between of N-benzoyl-staurosporine dose and hepatotoxicity is not significant ($p = 0.09$; Fisher's Exact).

[0030] Pharmacokinetic assessments were done in the clinical trial, but the two major metabolites of N-benzoyl-staurosporine could not be detected in the blood of most subjects because they are strongly bound to the serum protein α_1-acid glycoprotein (AGP). AGP is a heavily glycosylated protein that is synthesized primarily in the liver and functions as an acute phase response protein. Hochepied T *et al., Cytokine Growth Factor Rev* 14: 25-34 (2003); Israili ZH & Dayton PG, *Drug Met Rev* 33: 161-235 (2001). An analysis was done to see if AGP levels correlated with the occurrence of hepatotoxicity. The maximum AGP level from visit 3-5 was used for this analysis, and all participants in the clinical trial, not just those that consented to the pharmacogenetics analysis, were included. The mean maximum concentration of AGP among those that experienced hepatotoxicity was 109.7 mg/dl, which was significantly higher than 87.8 mg/dl, the mean maximum concentration for those that did not experience hepatotoxicity ($p=0.046$, ANOVA).

[0031] AGP is encoded by two genes, *ORM1* and *ORM2*, which are closely linked on chromosome 9q31-34.1. Webb GC *et al., Cytogenet Cell Genet* 47: 18-21 (1998). *ORM1* is highly polymorphic (Yuasa I *et al., Hum Genet* 99: 393-8 (1997)), and three SNPs in *ORM1* were interrogated in this trial (PG locus IDs 1831, 1832 and 1833). No association was seen between genotypes at the three *ORM1* loci examined and levels of AGP detected in the serum. See, Table 5.

Table 5
ORM1 SNPs do not associate with maximum AGP levels.

PG locus ID	AGP Max (p value, ANOVA)
1831	0.51
1832	0.07
1833	0.95

[0032] Furthermore, the *ORM1* SNPs interrogated in this analysis did not associate with hepatotoxicity. See, Table 6, below. To examine possible associations between the occurrence of hepatotoxicity and SNPs in *CYP2D6*, *CYP3A4*, *ABCB1* and *NR1I2* more thoroughly, each liver enzyme was examined independently. The maximum serum alanine aminotransferase and aspartate transaminase values recorded on visits 3-5 were analyzed using ANOVAs. No associations were found between SNPs in genes that contribute to the metabolism and distribution of N-benzoyl-staurosporine and the maximum elevation of either alanine aminotransferase or aspartate transaminase. No significant association was found between maximum alanine aminotransferase or aspartate transaminase levels and the *ORM1* polymorphisms.

Table 6
Association between hepatotoxicity, ALT Max and AST Max and 17 SNPs located in 6 different genes

Gene	PG Locus ID	Overall Hepatoxicity call (p value; Fisher's Exact)	ALT Max (p value; ANOVA)	AST Max (p value; ANOVA)
ABCB1	181	0.59	0.55	0.32
ABCB1	1006	1.00	0.63	0.24
ABCB1	1045	0.50	0.35	0.09
CD14	1708	1.00	0.87	0.90
CYP2D6	31	1.00	0.99	0.63
CYP2D6	211	1.00	0.85	0.66
CYP2D6	212	0.46	0.16	0.52
CYP2D6	213	0.22	0.17	0.59
CYP3A4	2325	0.72	0.38	0.49
IL1A	279	0.23	0.37	0.031
IL1A	302	0.29	0.38	0.029
NR1I2	2641	0.82	0.56	0.21
NR1I2	2642	0.84	0.28	0.86
NR1I2	2643	1.00	0.57	0.73
ORM1	1831	0.61	0.72	0.76
ORM1	1832	0.24	0.80	0.82
ORM1	1833	1.00	0.96	0.64

[0033] In summary, the data in Tables 5 and 6 did not provide evidence that the hepatotoxicity observed in the clinical trial was the result of varied exposure to N-benzoyl-staurosporine.

[0034] However, N-benzoyl-staurosporine is metabolized by *CYP3A4* and *CYP2D6*, and is a substrate of the p-glycoprotein pump, encoded by *ABCB1*. *CYP3A4* is not very polymorphic, and genetic variants rarely account for differences seen in the function of the protein. Spurdle AB *et al., Pharmacogenetics* 12: 355-66 (July 2002) and internal analysis. Also, transcription of *CYP3A4* is regulated by the pregnane X receptor, PXR (Goodwin B *et al., Annu Rev Pharmacol Toxicol* 42: 1-23 (2002)), which is encoded by a polymorphic gene called *NR1I2*. Accordingly, polymorphisms in the following genes that contribute to N-benzoyl-staurosporine metabolism were examined: *CYP2D6* (PG locus IDs 31, 211, 212, 213); *CYP3A4* (PG locus ID 2325); *NR1I2* (PG locus IDs 2641, 2642, 2643); and *ABCB1* (PG locus IDs 181, 1006, 1045). The occurrence

of hepatotoxicity in the clinical trial was not found to be associated with any of the polymorphisms interrogated in these four genes.

[0035] *Hepatotoxicity and genes associated with idiosyncratic mechanisms of liver injury.* Hepatotoxicity observed during the clinical stages of drug development is often associated with levels of exposure to the drug. However, some subjects may experience liver enzyme elevations because something about their intrahepatic milieu (levels of cytokines or enzymes that neutralize free radicals, for example) promotes toxicity of the compound. These mechanisms of liver toxicity are referred to as "idiosyncratic". Finding an association between genetic polymorphisms and hepatotoxicity arising from idiosyncratic mechanisms is not very likely because hepatotoxicity is a relatively rare event that can be influenced by multiple signaling pathways.

[0036] *Hepatotoxicity and genes associated with inflammatory response.* Polymorphisms in two genes that could contribute to acute phase responses in the liver, *CD14* and *IL1A*, were examined in this trial. *CD14* regulates release of inflammatory cytokines from Kuppfer cells (Jarvelainen HA *et al., Hepatology* 33: 1148-53 (2001)) and *IL1A* is an important mediator of responses to tissue damaging agents. Ramadori G & Christ B, *Semin Liver Dis* 19: 141-55 (1999). Two polymorphic loci in *IL1A* were interrogated in this trial (PG locus IDs 279 and 302) and three loci were investigated in *CD14* (PG locus IDs 2641, 2642 and 2643). No associations were found between the loci we studied and the overall hepatotoxicity call in the clinical trial. See, Table 5.

[0037] Next, SNPs in *IL1A* and *CD14* were analyzed for their possible association with elevations in each liver enzyme independently. The maximum serum aspartate transaminase or alanine aminotransferase value recorded between visits 3 and 5 was associated with genotypes at the loci of interest in *IL1A* and *CD14* using an ANOVA. No associations were found between alanine aminotransferase and SNPs in *IL1A* or *CD14*.

[0038] However, both *IL1A* SNPs, PG locus IDs 279 and 302, were associated with the maximum serum aspartate transaminase value recorded on visits 3, 4, or 5 ($p = 0.031$ and 0.029, respectively). PG locus ID 279, a C→T transition in the promoter of *IL1A* (position 549 in GenBank accession number X03833), has been reported to be in linkage disequilibrium with PG locus ID 302. Jouvenne P *et al., Eur Cytokine Netw* 10: 33-6 (1999). Our findings support strong linkage between these two loci (99.99%). PG locus ID 302 is a G→T base change in exon 5

(position 6282 in GenBank accession number X03833) that results in an amino acid substitution of alanine to serine. For each of these SNPs, the TT genotype is rare; only two individuals are TT for PG locus ID 279 and 302. For this reason, we analyzed TT homozygous individuals together with the GT (PG locus ID 302) or CT (PG locus ID 279) heterozygotes. Thus, for PG locus ID 279, subjects with a CC genotype were compared with subjects with a T at that locus (either CT or TT). For PG locus ID 279, the average maximum serum aspartate transaminase value for CC individuals was 37.1 U/L, while T (CT and TT) individuals had a significantly lower average maximum serum aspartate transaminase value of 23.1 U/L (p=0.0089, ANOVA). Likewise, for PG locus ID 302, subjects who were GG were compared to Ts (subjects who were either GT or TT). When the data were grouped using this approach, a much stronger association was seen between genotype at each locus and the occurrence of hepatotoxicity. For PG locus ID 302, GG individuals had an average maximum serum aspartate transaminase level of 36.6 U/L, which was significantly higher than 22.9 U/L, the average maximum serum aspartate transaminase level for T (CT and TT) individuals (p= 0.0097, ANOVA). The scatterplots in FIG. 1 and FIG. 2 show that the subjects with the highest maximum serum aspartate transaminase values while taking N-benzoyl-staurosporine were CC at PG locus ID 279 and GG at PG locus ID 302.

[0039] Because multiple SNPs were tested for their association with hepatotoxicity in the clinical trial, a correction factor was applied to the results. The Bonferroni correction method requires that p values be adjusted by a factor of 17 (the number of polymorphic SNPs interrogated in this trial). $\text{Bonferroni} = \frac{0.05}{\eta} = \frac{0.05}{17} = 0.0029$, where $\eta = PCK412\_number\_of\_tests$. Using this correction factor, no SNP with an association with $p \geq 0.0029$ is significant. Associations between *IL1A* polymorphisms and serum aspartate transaminase levels had p values of 0.0089 and 0.0097.

[0040] In summary, subjects who are CC at PG locus 279 and GG at PG locus 302 within the *IL1A* gene are more likely to experience elevated serum aspartate transaminase levels after taking N-benzoyl-staurosporine. While the average aspartate transaminase maximum for CC subjects was not over the upper limit of normal, a scatterplot of the data demonstrates that all but one of the subjects that had serum aspartate transaminase levels over the upper limit of normal were CC for PG locus ID 279 (FIG. 1) and GG for PG locus ID 302 (FIG. 2).

[0041] Drug-induced liver toxicity is often characterized by inflammation. Jaeschke H *et al.*, *Toxicol Sci* 65: 166-76 (2002). The interleukin 1 family of proteins is known to have multiple biological activities and are key regulators of the response to infection and injury. Like other proinflammatory cytokines, IL1A induces NF-κB activity, thereby increasing the transcription of cytokine-inducible genes. The effects of IL1A are mediated by the induction of other cytokines, including granulocyte colony-stimulating factor, tumor necrosis factor alpha (TNF), interleukin 6, interleukin 8, and platelet-derived growth factor. Paul WE, *Fundamental Immunology, Fourth Edition* (Lippingcott-Raven Publishers, Philadelphia, PA, 1999). Notably in mouse models, IL1A has been shown to act synergistically with TNF to induce liver injury. Nagakawa J *et al.*, *Immunopharmacol Immunotoxicol* 13: 485-98 (1991).

[0042] In addition to being associated with hepatotoxicity, the interleukin 1 family of proteins have been implicated in the induction of insulin secretion and stimulation of apoptosis in pancreatic β cells. Paul WE, *Fundamental Immunology, Fourth Edition* (Lippingcott-Raven Publishers, Philadelphia, PA, 1999). This is relevant for this trial since the clinical trial participants had either type I or type II diabetes. The production of IL1 by local inflammatory cells during the autoimmune process in insulin-dependent diabetes mellitus (type I diabetes) may contribute to the destruction of pancreatic β cells. Mandrup-Poulsen T *et al.*, *Cytokine* 5: 185-81 (1993). Interleukin 1 family members have been shown to induce the production of nitric oxide (NO) by pancreatic islets, and reports in the literature support a role for NO in diabetes development. Furthermore, treatment of the pancreatic β-cell-like line RIN-5AH with IL1A resulted in apoptosis and necrosis within 4 hours after treatment. Vassiliadis S *et al.*, *Mediators Inflamm* 8: 85-91 (1999). Interestingly, IL1A was shown to induce the expression of PKC in RIN-5AH cells by 30%. Therefore, in addition to influencing hepatotoxicity, IL1A may influence the efficacy of N-benzoyl-staurosporine in diabetic patients.

[0043] All references cited herein are incorporated herein by reference in their entirety and for all purposes to the same extent as if each individual publication or patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety for all purposes. In addition, all GenBank accession numbers, Unigene Cluster numbers and protein accession numbers cited herein are incorporated herein by reference in their entirety and for all

purposes to the same extent as if each such number was specifically and individually indicated to be incorporated by reference in its entirety for all purposes.

[0044] The present invention is not to be limited in terms of the particular embodiments described in this application, which are intended as single illustrations of individual aspects of the invention. Many modifications and variations of this invention can be made without departing from its spirit and scope, as will be apparent to those skilled in the art. Functionally equivalent methods and apparatus within the scope of the invention, in addition to those enumerated herein, will be apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications and variations are intended to fall within the scope of the appended claims. The present invention is to be limited only by the terms of the appended claims, along with the full scope of equivalents to which such claims are entitled.

CLAIMS

We claim:

1. A method for predicting hepatotoxicity in a subject, comprising the steps of:
   (a) obtaining the genotype of a subject at an *IL1A* genetic locus predictive of hepatotoxicity following administration of a staurosporine derivative; and
   (b) determining whether the subject is at risk for hepatotoxicity following administration of the staurosporine derivative.

2. The method of claim 1, wherein the *IL1A* genetic locus is PG locus ID 279.

3. The method of claim 2, wherein a CC genotype at the PG locus ID 279 is predictive of a high risk of hepatotoxicity.

4. The method of claim 2, wherein a CT or TT genotype at the PG locus ID 279 is predictive of a low or average risk of hepatotoxicity.

5. The method of claim 1, wherein the *IL1A* genetic locus is PG locus ID 302.

6. The method of claim 5, wherein a GG genotype at the PG locus ID 302 is predictive of a high risk of hepatotoxicity.

7. The method of claim 5, wherein a CT or TT genotype at the PG locus ID 302 is predictive of a low or average risk of hepatotoxicity.

8. An improved method for treating a diabetic condition with at staurosporine derivative, comprising the steps of:
   (a) obtaining the genotype of a subject to be treated at an *IL1A* genetic locus predictive of hepatotoxicity following administration of the staurosporine derivative;
   (b) administering the staurosporine derivative to the subject.

9. A method for choosing a subject for inclusion in a clinical trial for determining the efficacy of treatment with a staurosporine derivative, comprising the steps of:

(a) obtaining the genotype of a subject at an *IL1A* genetic locus predictive of hepatotoxicity following administration of a staurosporine derivative; and

(b) then:

(i) including the subject in the trial if the genotype indicates a low or average risk of hepatotoxicity; or

(ii) excluding the subject from the trial if the genotype indicates a high risk of hepatotoxicity.

10. A kit for use in predicting hepatotoxicity, comprising:

(a) a reagent for detecting a genetic polymorphism in the *IL1A* gene that is biomarker of staurosporine derivative-mediated hepatotoxicity;

(b) a container for the reagent; and

(c) a written product on or in the container describing the use of the biomarker in predicting staurosporine derivative-mediated hepatotoxicity in subjects.

11. The kit of claim 10, wherein the *IL1A* genetic locus is PG locus ID 279.

12. The kit of claim 10, wherein the *IL1A* genetic locus is PG locus ID 302.

13. The kit of claim 10, wherein the reagent is a set of primer pairs that hybridize to a polynucleotide on either the side of the genetic polymorphism and which define a nucleotide region that spans the genetic polymorphism.

ABSTRACT

IL1A or a gene located near *IL1A* on chromosome 2q14 may contribute to hepatotoxicity, as measured by increased serum levels of aspartate transaminase, during N-benzoyl-staurosporine treatment for macular edema. Accordingly, genetic polymorphisms in the *IL1A* gene are useful as biomarkers for predicting staurosporine derivative -mediated hepatotoxicity.

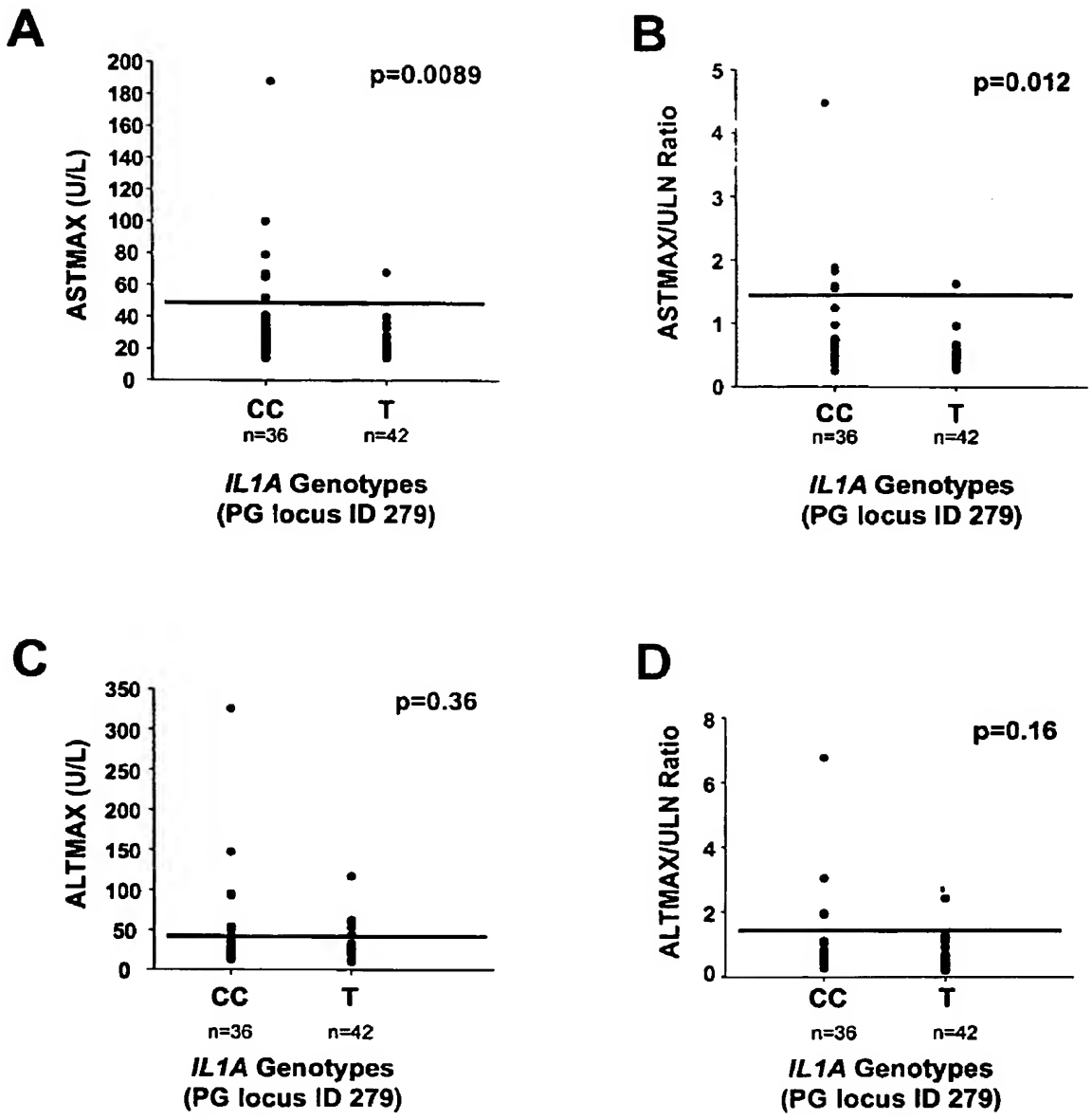

FIG. 1
AST/ALT Maximum Levels v *IL1A* (PG locus ID 279)

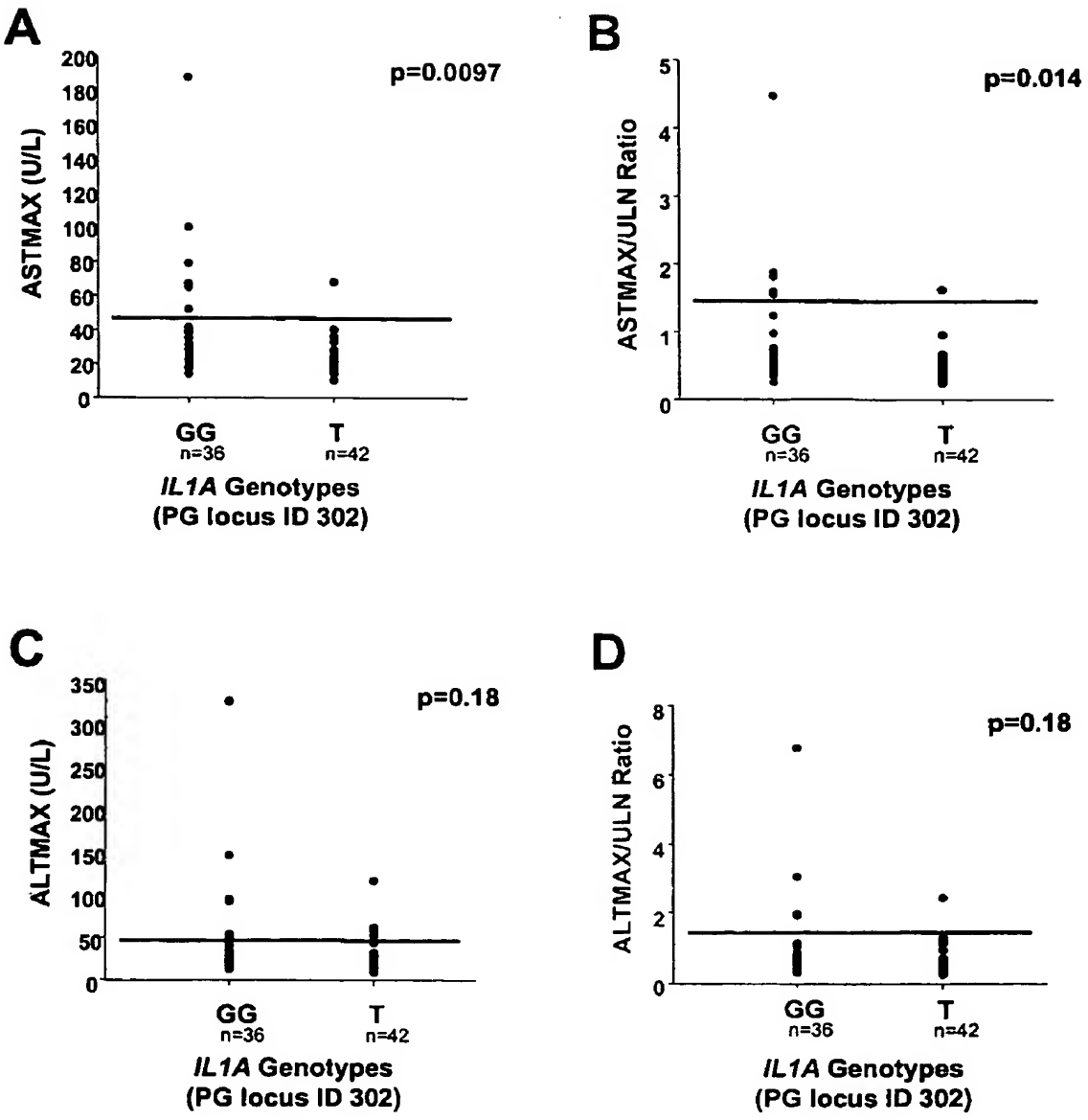

FIG. 2
AST/ALT Maximum Levels v *IL1A* (PG locus ID 302)

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